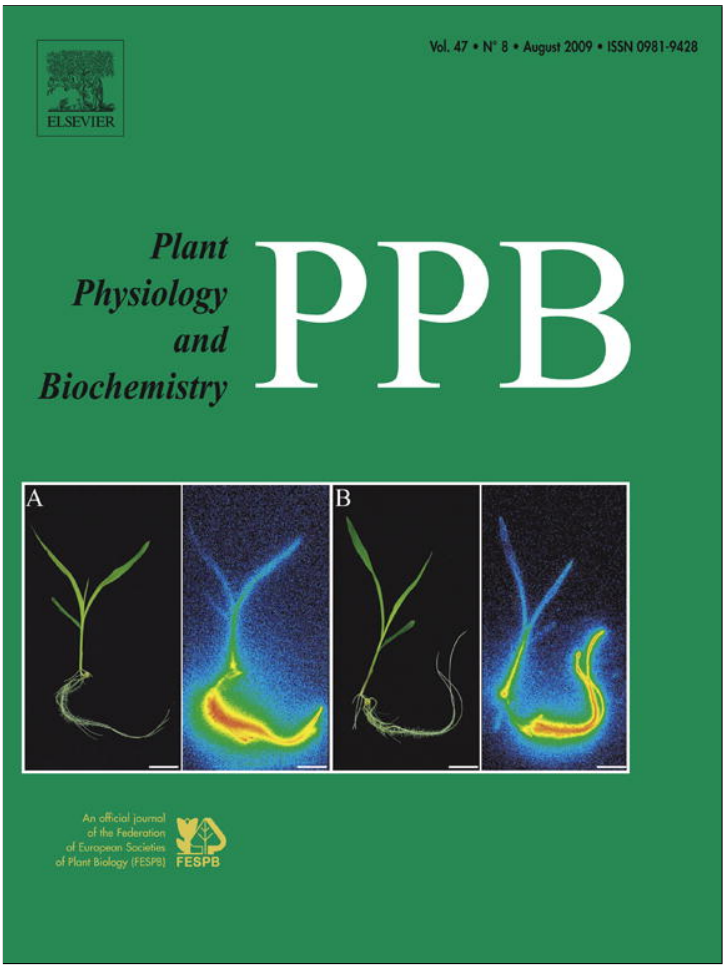

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Research article

# Differential regulation of *SERK*, *LEC1-Like* and *Pathogenesis-Related* genes during indirect secondary somatic embryogenesis in grapevine

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ABSTRACT

A culture model was developed in *Vitis vinifera* L., cultivar 'Chardonnay' for studying SE (Somatic Embryogenesis). The auxin 2,4-D (2,4-Dichlorophenoxyacetic acid) was used to induce indirect secondary embryogenesis at a high rate, starting from embryos derived from embryogenic cultures previously obtained. Cotyledonary embryos were shown to be more responsive to SE induction than embryos at the torpedo-stage and were used for molecular analyses. The expression of *SERK* (*Somatic Embryogenesis Receptor Kinase*), *L1L* (*Leafy Cotyledon1 Like*) and a set of *PR* (*Pathogenesis-Related*) genes was monitored during the whole SE process. *VvSERK1*, *VvSERK2* and *VvL1L* were down-regulated by the 2,4-D treatment but expressed in embryonic tissues. On the contrary, *VvPR1*, *VvPR8*, *VvPR10.1* and *VvPR10.3* were strongly up-regulated by the 2,4-D treatment, and their transcripts were not or only weakly detected in clusters of secondary embryos. *VvSERK3*, *VvPR3* and *VvPR10.2* were more stably expressed in all tissues examined. The discussion deals with the putative role of the different genes in grapevine SE.



## 1. Introduction

Somatic embryos are the most suitable tissues for transformation of grapevine, transgenesis providing new opportunities for improving this world-wide important crop [44]. Unfortunately, economically important cultivars remain recalcitrant to in vitro biotechnologies. Although SE (Somatic Embryogenesis) can be induced from diverse floral and vegetative explants, embryos are produced at a rather low rate [10,23,39,43,46,51]. Secondary embryogenesis is used to increase the simultaneous production of numerous embryos, however limited to a handful of cultivars [9,42]. Much progress is therefore expected for developing SE in grapevine. The elucidation of molecular events occurring during the embryogenic process would certainly contribute to this purpose.

Due to technical convenience, embryogenesis has especially been studied in model plants such as *Daucus carota* and *Arabidopsis thaliana*, mutants having brought important information about this process [16,21,53]. Numerous genes have been shown to be regulated during zygotic and somatic embryogenesis: genes encoding transcription factors and cell cycle regulators, homeotic genes, hormone-inducible genes, maturation genes, and several genes encoding extracellular proteins such as AGPs (ArabinoGalactan Proteins) or secreted chitinases and lipid transfer proteins [7]. Recently, transcriptomic studies of chicory and wheat embryogenesis highlighted the regulation of a wide set of genes related to several biosynthetic pathways [36,64]. Nevertheless, the specific role of most genes as well as their functional connections remain to be determined. Furthermore, while embryo morphogenesis and maturation become more and more clear, very few is known about the molecular events involved in the shift of a somatic cell to an embryogenic state. *SERK* (*Somatic Embryogenesis Receptor Kinase*) is the best characterized gene involved in early embryogenesis. First identified in carrot, *SERK* was found to be especially expressed in embryonic tissues of many dicots and monocots [24,29,61,63,65]. The ectopic expression of *SERK1* in *Arabidopsis* was shown to result in the enhancement of embryonic cell formation, highlighting its crucial role during embryogenesis induction [24]. *LEC* (*Leafy Cotyledon*) and *L1L* (*Leafy Cotyledon1 Like*) genes that code for transcription factors have also been shown to play a significant role in early steps of plant zygotic and somatic embryogenesis

*Abbreviations:* 2,4-D, 2,4-Dichlorophenoxyacetic acid; AGPs, ArabinoGalactan Proteins; BAP, 6-BenzylAminoPurine; IAA, Indole-3-Acetic Acid; L1L, Leafy Cotyledon1 Like; NAA, Napthaleneacetic Acid; NOA, 2-NaphtOxyacetic Acid; PR, Pathogenesis-Related; SE, Somatic Embryogenesis; SERK, Somatic Embryogenesis Receptor Kinase.

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[1,15,22,35]. Recently, we characterized three *SERK* genes (*VvSERK1*, *VvSERK2*, *VvSERK3*) and one *L1L* gene (*VvL1L*) expressed in a stabilized embryogenic cell line of grapevine [62].

SE is usually obtained from tissues subjected to varied stresses or treated with hormones, mainly the synthetic auxin 2,4-D (2,4-Dichlorophenoxyacetic acid) [16,21]. This auxin analog is known for its herbicide activity, inducing a general oxidative stress in treated seedlings, followed by protein degradation that probably results in phytotoxicity [60]. A short treatment of plant tissues is able to trigger the reprogramming of somatic cells toward embryogenesis, probably as a consequence of stress induction together with the modification of internal auxin content [53]. As a result, defence proteins can be synthesized, further suggesting that SE is an adaptative process of plant cells to stress [16]. Pathogenesis-Related (PR) proteins are part of the plant defence responses. They are divided among different classes based on their structure, serological relationships and biological functions, and are usually observed upon biotic and abiotic stresses [69]. Although some PR proteins clearly show an anti-microbial activity, many reports deal with a probable additional role in plant development, including SE. For example, most proteins secreted during embryogenesis code for chitinases or glucanases [53]. The accumulation of β-1,3-glucanases, acidic chitinases and osmotin-like proteins is correlated with SE, in chicory [26,27]. Chitinases belong to a large family of enzymes able to hydrolyse the β-1,4-glycoside bond present in biopolymers of N-acetylglucosamine, such as chitin, therefore being thought to degrade fungi at the time of a pathogen attack. In addition, chitinases can cleave plant endogenous substrates such as cell wall AGPs, producing oligosaccharides that could play a role in development as signal molecules [32]. A chitinase has been shown to rescue the carrot mutant *tsl1*, arrested at the globular stage of embryonic development, probably by the release of AGPs derived molecules able to stimulate SE [11,68]. Likewise, although concluding experiments suggest a defence role of PR10 proteins as ribonucleases, these intracellular proteins are probably assigned to additional functions in plant growth and development [38]. Betv1, the major allergenic PR10 protein of birch, is able to bind such diverse molecules as fatty acids, flavonoids, cytokinins and brassinosteroids, probably playing a role in the cytoplasmic transport of hydrophobic ligands and interfering with hormonal regulation in plants [17,33,40,45].

In the following study, we focused on the development of an in vitro culture model allowing the recovery of large amounts of synchronized somatic embryos, with the aim of stuying molecular events of grapevine embryogenesis. We monitored the expression of *VvSERK1*, *VvSERK2*, *VvSERK3* and *VvL1L* on the one hand, and of a set of defence genes, on the other hand: a *PR3* gene and a *PR8* gene respectively coding for a typical basic vacuolar chitinase and an acidic secreted chitinase, three *PR10* genes and a *PR1* gene coding for a secreted protein with an unknown function.

## 2. Results

### 2.1. Induction of secondary embryogenesis

Embryogenic cultures previously obtained from the cultivar 'Chardonnay' were used to produce numerous embryos at the same time (Fig. 1A). For inducing secondary embryogenesis, embryos

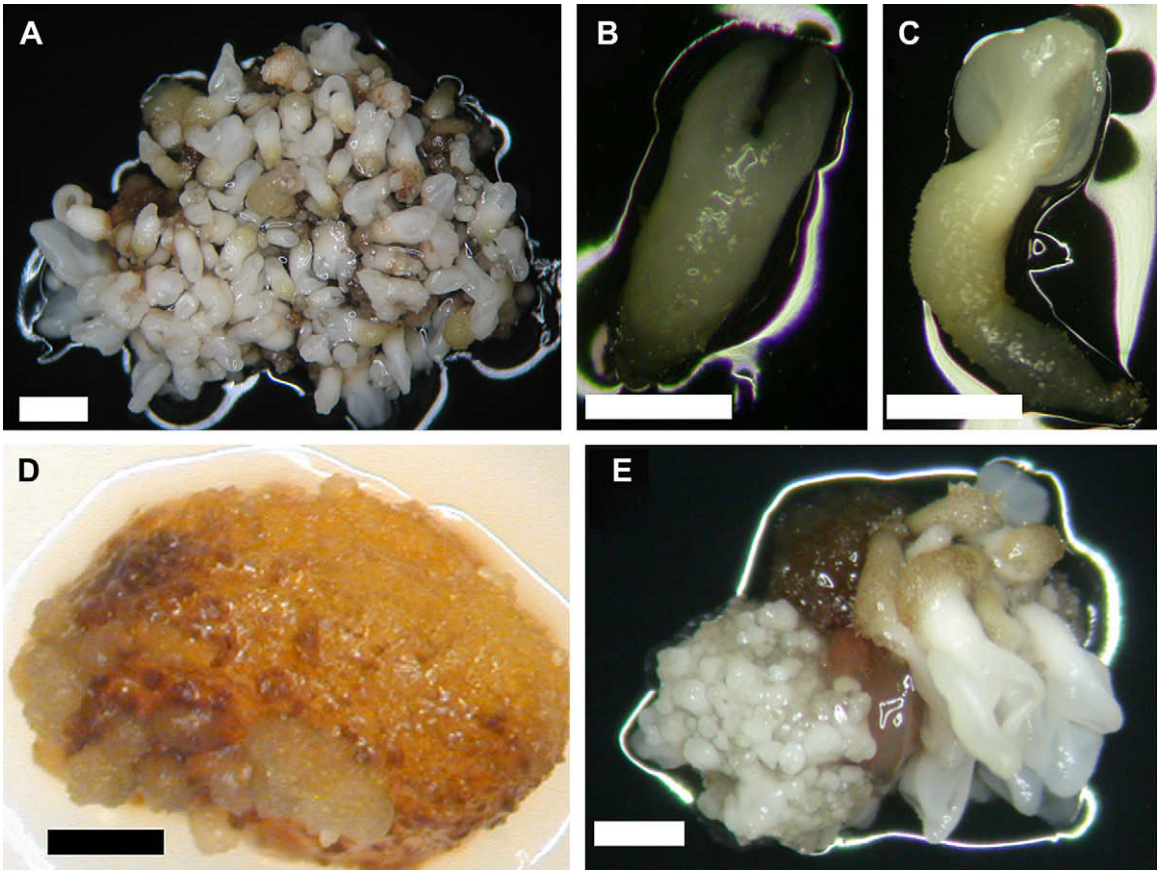


**Fig. 1.** Induction of secondary embryogenesis from somatic embryos of *Vitis vinifera* cv. 'Chardonnnay'. (A) Starting embryogenic cultures providing numerous embryos at the same time; (B) Embryo at the torpedo-stage; (C) Embryo at the cotyledonary-stage; (D) Compact yellowish callus obtained from an embryo after culture on a medium containing 2,4-D; (E) Secondary embryogenic clusters composed of embryos at varied development stages on the surface of an embryogenic callus. Bars = 2 mm (A), 1 mm (C, D and E) and 0.5 mm (B).

either at the torpedo- (Fig. 1B) or at the cotyledonary-stage (Fig. 1C) were selected. To assess secondary embryogenesis efficiency, four independant experiments were performed involving a total of 346 embryos, separately plated on a culture medium with 2,4-D. After 3 weeks, a compact and yellowish callus was obtained from every embryo (Fig. 1D). After their transfer onto the medium A, calli stopped growing and progressively turned brown. Clusters of secondary embryos appeared on some calli within an additional culture period of 1–4 months on the medium A (Fig. 1E): they were composed of a mix of very small and translucent globular structures and of opaque white embryos at varied developmental stages. In average, 29% of the plated embryos at the torpedo-stage and 68% of the embryos at the cotyledonary-stage gave rise to secondary embryogenic calli (Table 1). In a further experiment, embryos at the cotyledonary-stage were used to induce secondary embryogenesis at a high rate and perform molecular analyses.

### 2.2. Identification of PCR products

PCR products were sequenced and compared to the whole *Vitis vinifera* genome recently available in the Genoscope databank. When required, a comparison with sequences of other flowering plants was performed, using the BLAST program of the National Center for Biotechnical Information (NCBI) databank. The sequences obtained with primers specific of *VvSERK1*, *VvSERK2*, *VvSERK3* and *VvL1L* cDNAs corresponded to the annotations GSVIVT00001777001 on the chromosome 18, GSVIVT00019412001 on the chromosome 7, GSVIVT00009544001 on the chromosome 12 and GSVIVT00010958001 on the chromosome 10, respectively. They were identical to those previously described [62]. As expected, the sequence amplified with specific primers of a *VvPR1* cDNA was found to be identical to the accession AJ536326; it was located on the chromosome 3. The sequence amplified with specific primers of a *VvPR10.2* cDNA was identical to that of the *VvPR10.2* gene of the cultivar 'Ugni blanc' (AJ291704) and was located on the chromosome 5. The sequence amplified with the primers corresponding to a *VvPR3* cDNA was found to be identical to the *VCHI1b* gene isolated from 'Pinot noir' [4] and to *VvChit1a* from 'Ugni blanc' [58], located on the chromosome 3 and coding for the same basic vacuolar class I chitinase (AJ291505). The sequence amplified with primers corresponding to a *VvPR8* cDNA showed 97% homology with a class III chitinase gene of the cultivar 'Koshu' (AB105374) and 93% with a class III chitinase gene of 'Pinot noir' and 'Ugni blanc' (AJ291507). This *VvPR8* gene was found to be located on a contig not linked to a known chromosome in the *V. vinifera* genome.

Two 244 bp long different sequences were amplified with primers previously described for the specific amplification of a *VvPR10.1* cDNA (AJ291705) [57]. A first sequence showed 99.6% homology with the Genoscope annotation GSVIVT00033089001 related to *VvPR10.1*, while a second sequence showed 100% homology with a genomic sequence located on the chromosome 5 at a location clearly different from that of *VvPR10.1*. At this location, two genomic sequences were annotated as separate very short transcripts, but not as a single *VvPR10* gene. However it corresponded to grapevine ESTs in the banks of the Istituto Agrario di San Michele all'Adige (IASMA), the Genoscope and the Dana-Farber Cancer Institute (DFCI): ISMAAEST003738, CDMVvT00000963001 and TC74380, respectively, and was recognized as the incomplete cDNA of a putative *VvPR10* gene in the NCBI bank. The identification of the complete sequence was performed using a RACE-PCR method. A set of primers was specifically designed for the elongation and amplification of this putative *VvPR10* cDNA. A 799 bp long sequence, colinear with the first sequence of 244 bp, was obtained and sequenced. The complete sequence included a 480 bp long region coding for a polypeptide of 159 amino-acid residues, a 5′-UTR of 80 bp and a 3′-UTR of 239 bp. The full gene corresponding sequence deduced from the Genoscope databank is interrupted by an intron of 129 bp. The nucleotidic sequence and the predicted translation product present the characteristics of genes and proteins related to the birch major pollen allergen Betv1 [28]: a conserved glycine-rich "P-loop" motif GXGGXGXXK at the position 47–55 associated with a putative hydrophobic cavity at the position 89–121 and an intron position at codon 62 (Fig. 2A), as well as a calculated molecular mass of 17.4 kDa and a theoretical isoelectric point of 6.3. The full amino acid sequence shows the maximal percentage of identity (96%) with *Vitis quinquangularis* PR10.3 (ABD78555), a high similarity with *Vitis pseudoreticulata* PR10 proteins and is more distinct from *V. vinifera* PR10.1 and PR10.2 (Fig. 2B and C). According to the nomenclature recommendations, the corresponding gene has been named *VvPR10.3* and the cDNA sequence has been submittted for publication in the GenBank available to EMBL in Europe and the DNA Data Bank of Japan (NCBI): EU379313 coding for the protein ACA58119. The amplification of two distinct *VvPR10* cDNAs with primers previously designed for the specific amplification of a *VvPR10.1* transcript [57] is explained by a high homology of *VvPR10.1* and *VvPR10.3* sequences at the locations of primer hybridization. New primers were then designed for the separate amplification of *VvPR10.1* and *VvPR10.3* (Fig. 3).

**Table 1**
Efficiency of secondary embryogenesis. Somatic embryos either at the torpedo-stage (experiments A and B) or at the cotyledonary-stage (experiments C and D) were separately treated for inducing secondary somatic embryogenesis.

| Embryo stage | Torpedo | | Cotyledonary | |
|---|---|---|---|---|
| Experiment | A | B | C | D |
| Number of plated embryos | 79 | 117 | 85 | 65 |
| Number of secondary embryogenic calli | 23 | 34 | 65 | 39 |
| Secondary embryogenesis efficiency % | 29.11 | 29.06 | 76.47 | 60.00 |
| Average efficiency[a] % | **29.09** ± 0.04 | | **68.24** ± 11.65 | |

[a] Average efficiency = mean of secondary embryogenesis efficiencies obtained in separate experiments and calculated as the number of secondary embryogenic calli recovered from 100 tested embryos, ±standard deviation.

### 2.3. Gene expression

We recorded the expression of some *PR* genes during the whole process of secondary embryogenesis as well as that of *SERK* and *L1L* genes usually involved in plant embryogenesis (Fig. 4). *Actin* was used as a reference gene. The transcription of all the genes except *VvPR1* was detected in cotyledonary embryos (E) at the beginning of the experiment, although it was very weak for *VvPR8,* which encodes a class III chitinase. Then, the expression of *VvPR10.2* was scarcely increased by the 2,4-D treatment, while that of *VvPR1*, *VvPR8*, *VvPR10.1* and *VvPR10.3* was clearly up-regulated (C1). Calli expressed every *PR* gene at the same level after being transferred onto the medium A for one week, suggesting that gene expression was not immediately affected by the removal of 2,4-D (C2). Later, *VvPR3* and *VvPR10* transcripts were detected in calli, but not *VvPR1* nor *VvPR8* transcripts (NEC and EC). In clusters of secondary embryos detached from embryogenic calli (CSE), the expression of *VvPR3* and *VvPR10* genes was detectable at a lower rate than in embryogenic calli, whereas that of *VvPR1* and *VvPR8* was not detected at all. On the whole, *PR* genes were similarly expressed in secondary embryogenic clusters (CSE) and in embryos at the beginning of the experiment (E). In germinated embryos (GE), these genes were expressed at the same or at a weaker level than in cotyledonary embryos (E) and secondary embryogenic clusters (CSE). Together, these results show that *PR* genes were differently induced by the 2,4-D treatment, *VvPR1*, *VvPR8, VvPR10.1* and

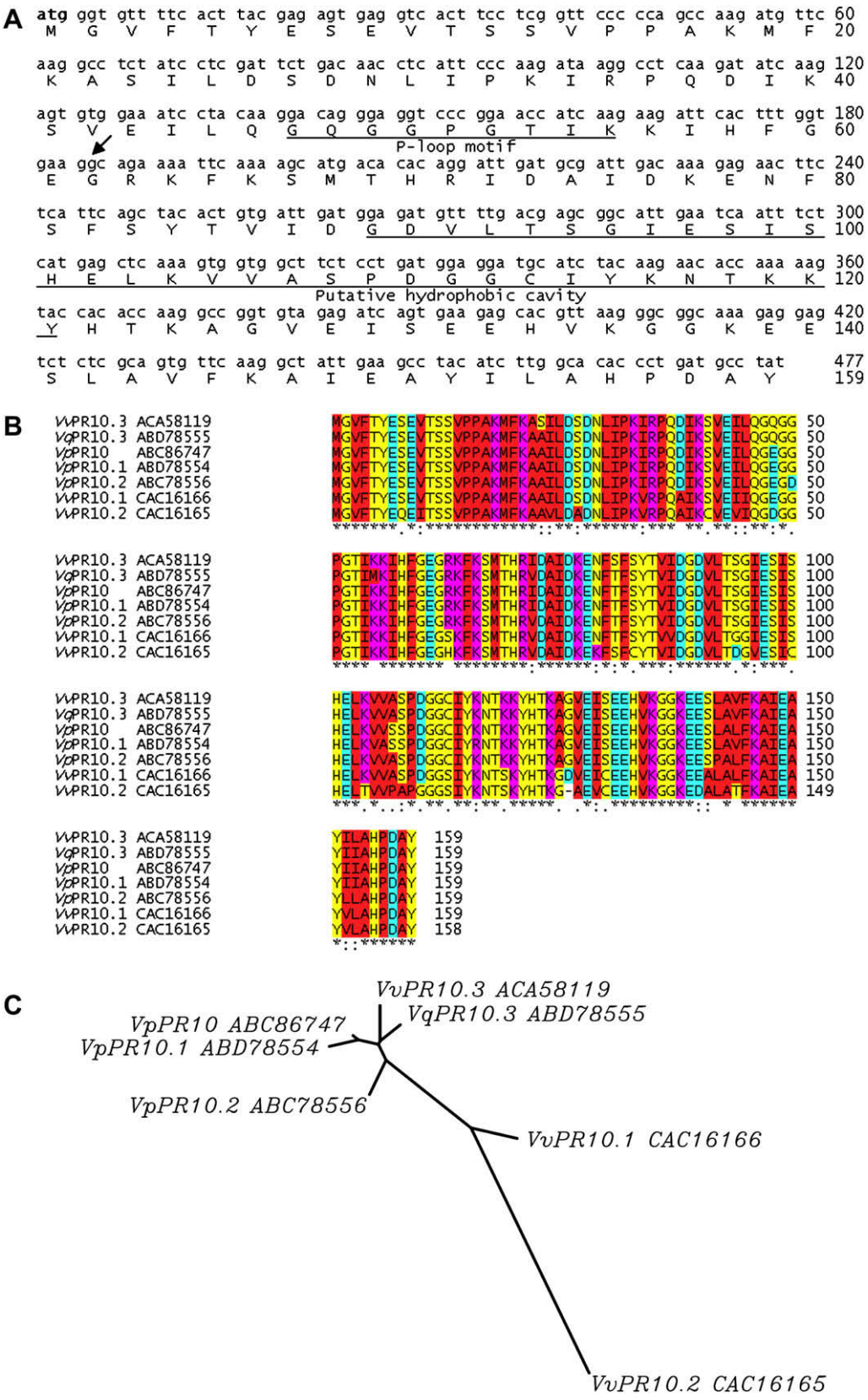


**Fig. 2.** Sequence analysis of *Vitis vinifera* PR10.3 (ACA58119) and comparison with other PR10 proteins in *Vitis sp.* (A) Nucleotidic sequence and predicted translation product of *VvPR10.3*. The main characteristics of Betv1-related proteins are presented: a P-loop motif at the amino-acid position 47–55, a hydrophobic cavity at the amino-acid position 89–121 and an intron position indicated by an arrow at the codon 62. Bold letters indicate the ATG initiation codon; (B) Alignment with ClustalW of the amino-acid sequence of *Vitis vinifera* PR10.3, with the most related PR10 proteins: *Vitis quinquangularis* PR10.3 (ABD78555), *Vitis pseudoreticulata* PR10 (ABC86747), PR10.1 (ABD78554) and PR10.2 (ABC78556), and *Vitis vinifera* PR10.1 (CAC16166) and PR10.2 (CAC16165); (C) Unrooted dendrogram showing the approximate distance of *Vitis vinifera* PR10.3 with the most related *Vitis* PR10 sequences.

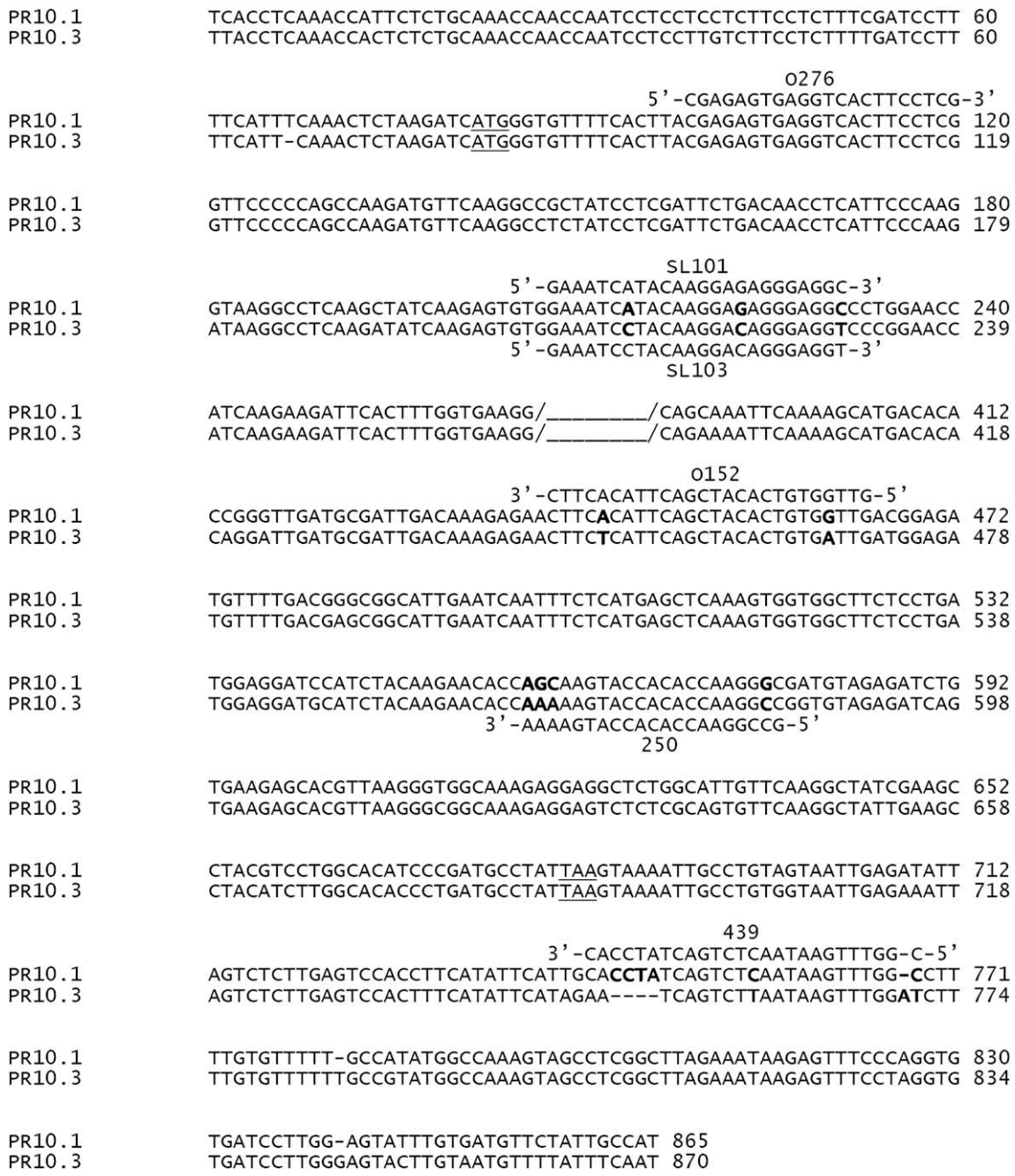


**Fig. 3.** Hybridization of three primer pairs with *VvPR10.1* and *VvPR10.3* cDNAs: '0152–0276' detecting the two cDNAs, 'SL101–439' for the specific detection of *VvPR10.1*, and 'SL103–250' for the specific detection of *VvPR10.3*. Bold types indicate nucleotidic differences at the hybridization sites. The ATG initiation and TAA terminator codons are underlined. Slashes indicate the intron position.

*VvPR10.3* being very responsive to the treatment, contrary to *VvPR3* and *VvPR10.2*. Further, only *VvPR3* and *VvPR10* transcripts were detected in calli at the latest culture steps (NEC and EC). Contrary to defence genes, *VvSERK1*, *VvSERK2* and *VvL1L* expression was recorded at the highest level in cotyledonary embryos at the beginning of the embryogenic process (E) and in secondary embryogenic clusters (CSE). It was slightly down-regulated by the 2,4-D treatment (C1) and remained low in calli (C2, NEC and EC). By contrast, *VvSERK3* expression was rather stable during the experiment. In germinated embryos (GE), the expression of *SERK* and *L1L* genes was similar or lowered compared to cotyledonary embryos (E) or secondary embryogenic clusters (CSE).

## 3. Discussion

### 3.1. Induction of secondary embryogenesis

Embryogenic cultures provided us with numerous secondary embryos that we brought to an additional embryogenic cycle. Embryos at the torpedo- or cotyledonary-stage were separately plated on a medium containing 2,4-D to form calli. After being transferred onto the medium A, some calli gave rise to clusters of secondary embryos within a period of 1–4 months. SE was therefore an indirect process. As a result, 29–76% of the plated embryos were led to secondary embryogenesis. In previous experiments

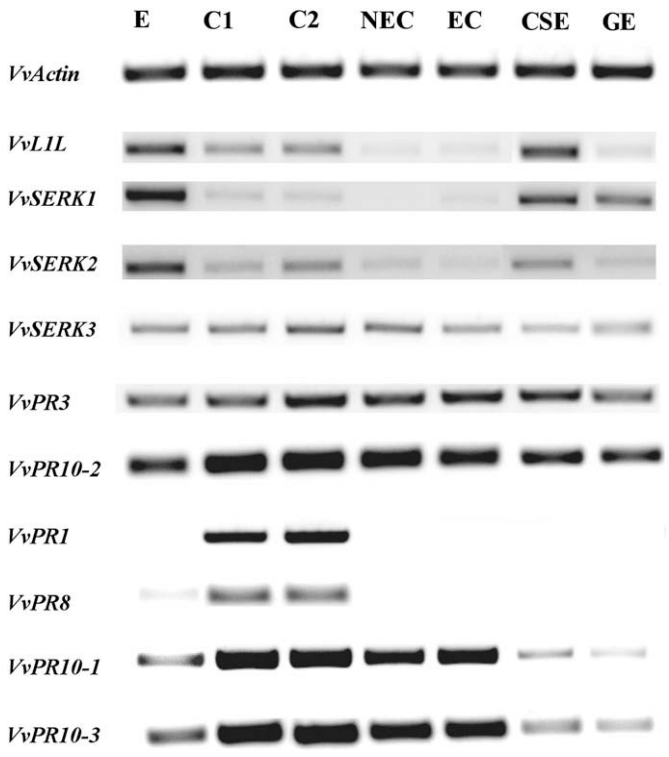


**Fig. 4.** Expression of *Vitis vinifera Actin*, *L1L*, *SERK*, and *PR* genes, during induction of secondary embryogenesis. E: cotyledonary embryos at the beginning of the experiment; C1: calli developed from embryos on the medium E96; C2: calli after one week subculture on the medium A; NEC: non-embryogenic calli and EC: embryogenic calli cleared of embryogenic clusters, after 12 weeks on the medium A (3 subcultures); CSE: clusters of secondary embryos; GE: germinated embryos.

aimed to induce primary embryogenesis, we showed that only 6% of nodal explants from the cultivar 'Chardonnay' gave rise to embryogenic calli [39]. This result is in accordance with other studies showing that secondary embryogenesis is more effective than primary embryogenesis in grapevine [9,42], just as in other plant species [54]. It further suggests that embryos are more suitable than vegetative organs for SE induction. Futhermore, the use of zygotic embryos has made SE possible for plant species considered to be recalcitrant [21]. Embryos at the cotyledonary-stage gave around 2.3 fold more embryogenic calli than younger embryos, showing that they were more responsive to SE induction. Likewise, immature zygotic embryos of *Arabidopsis*, with fully-developed cotyledons, gave better results than younger embryos in secondary SE experiments [20]. On the contrary, hot pepper embryos at the globular- and heart-stage were found to have a higher potential than cotyledonary embryos for direct SE [48]. Different responses can thus be obtained depending on the species and/or protocol. Our protocol induced indirect SE: a cell dedifferentiation step followed by cell proliferation resulted in a growing callus before the development of secondary embryos. *Arabidopsis* zygotic embryos at the cotyledonary-stage can also be led to indirect SE; secondary embryos develop from proliferating cells arising from the cotyledons, but never from the other parts of the embryo, showing that cotyledons contain those cells able to enter an embryogenic program [55]. This further suggests that well-developed embryos are quite suitable for inducing SE.

### 3.2. Expression of SERK and L1L genes during embryogenesis

The ability of differentiated tissues to generate embryos, termed 'embryogenic competence', is often induced by a stress or a treatment with hormones [16,21]. Embryogenic competence is generally accompanied by an enhanced expression of *SERK* genes coding for transmembrane somatic embryogenesis receptor kinases. *SERK* expression has thus been recorded in zygotic and somatic embryos of numerous plant species [24,29,61,63,65]. Moreover, *AtSERK1* overexpression has been shown to increase embryogenesis in *Arabidopsis* [24]. *SERK* is therefore seen as a ubiquitous marker of plant embryogenic competence. *LEC* and *L1L* genes have also been shown to interfer with early plant embryogenesis [1,15,22,35]. We previously characterized three *SERK* genes in grapevine: *VvSERK1*, *VvSERK2* and *VvSERK3*, homologous to *AtSERK1*, *AtSERK2* and *AtSERK3*, as well as a *L1L* gene homologous to *AtL1L*: *VvL1L*; these four genes were shown to be expressed in a stabilized embryogenic cell line of Chardonnay [62]. During secondary SE, VvSERK1, VvSERK2 and VvL1L expression was detected in embryos at the beginning of the experiment. It was then down-regulated by the 2,4-D treatment. Their transcription remained low in calli at every culture step, was enhanced in secondary embryogenic clusters and further decreased during conversion of embryos into plantlets. The highest transcription level of *VvSERK1, VvSERK2* and *VvL1L* was found in embryos at the beginning of the experiment and in secondary embryogenic clusters, suggesting a role for the encoded proteins in grapevine embryogenesis. On the contrary, *VvSERK3* transcription was rather stable in all tissues examined. These results are in accordance with studies on *Arabidopsis*, which showed that *AtSERK1* and *AtSERK2* act redundantly in embryogenesis [8], while *AtSERK3* is rather involved in plant immunity [25] and brassinosteroid signaling [37,49]. *AtL1L* has also been shown to be specifically expressed in early and late stages of embryo development in *Arabidopsis* [35].

*VvSERK1*, *VvSERK2* and *VvL1L* expression was identical in non-embryogenic calli and embryogenic calli cleared of embryogenic clusters. Embryogenic competence is thought to be limited to a small subset of plant cells. In carrot, they derive from cytoplasmic rich cells originating from the provascular elements of the hypocotyl and proliferating after the 2,4-D treatment [63]. In *Dactylis*, small cytoplasmic rich competent cells are located close to the vascular bundles [65]. In *Arabidopsis* plant lines expressing a SERK1-YFP fusion protein, a prolonged 2,4-D application on seedling hypocotyls was shown to result in the multiplication of procambium cells specifically expressing *AtSERK1*, as visualized by fluorescence monitoring [34]. Likewise, in our culture model, the shift toward 'embryogenic competence' could firstly concern only a very small part of the callus cells, led to the execution of a new genetic program. These cells, at the origin of embryogenic clusters, could express *SERK* and *L1L* genes as an evidence of embryogenic competence acquisition, while the rest of the callus would not. Therefore, a very weak modification of transcription, due to scarce competent cells into the whole callus, would only be detectable by histological localisation methods.

*VvSERK1*, *VvSERK2* and *VvL1L* expression slightly decreased following the 2,4-D treatment and remained low in calli. In control experiments, when embryos were continuously cultured on the medium A, being not previously treated by 2,4-D, or when 2,4-D was replaced with a weak auxin such as NAA (NaphtaleneAcetic Acid), no secondary embryogenesis was observed, strongly suggesting that 2,4-D is necessary for inducing SE (data not shown). The synthetic auxin 2,4-D is thought to be crucial for the acquisition of embryogenic competence, acting as a shock triggering cell division before the development of somatic embryos [7,16]. However, although some *SERK* genes have been tightly linked to embryogenic competence, their expression is not always observed in freshly 2,4-D-treated tissues. When wheat leaf bases are submitted to a 2,4-D treatment, *SERK* is firstly down-regulated, being strongly expressed only at the time of embryo differentiation [64]. In other respects,

a high expression of *SERK* is not restricted to embryogenic competent cells. In *Medicago truncatula*, a treatment with NAA and BAP was found to stimulate *SERK* expression in two different cell lines, one being embryogenic and the other not [50]. Likewise, in sunflower, *SERK* is up-regulated in immature zygotic embryos driven either to organogenesis or to somatic embryogenesis, suggesting that *SERK* expression alone is rather correlated to an intense mitotic activity [67]. In our culture system, *VvSERK1*, *VvSERK2* and *VvL1L* expression was the highest in cotyledonary embryos as well as in secondary embryogenic clusters, showing that the corresponding proteins could play a role in the early development of grapevine.

### 3.3. Characterization of a VvPR10.3 gene

Using primers designed for the amplification of *VvPR10.1*, we detected a *VvPR10.3* transcript. We determined that it probably codes for a protein structurally related to the major food and pollen allergens of trees, close to birch Betv1 [3] and very similar to *V. quinquangularis* PR10.3 (ABD78555). Plant PR10 proteins show a very conservative sequence and a similar three-dimensional (3D) structure. They possess a characteristic P-loop (Phosphate-binding motif) sequence and a hydrophobic cavity, making the 'Betv1-signature' [28]. The P-loop is presumed to function as a binding site for ATP or GTP, involved in RNase and anti-microbial activity, and the Y-shaped hydrophobic cavity is able to bind hydrophobic molecules such as fatty acids, flavonoids, cytokinins and brassinosteroids for their probable intracellular transport [17,33,38,40,45]. We found that the nucleotidic sequence of *VvPR10.3* is very homologous to that of *VvPR10.1* and *VvPR10.2*, suggesting that all three genes could originate from a common ancestral sequence, as proposed for *PR10* genes of birch, apple and passiflora [2,18,70].

### 3.4. Induction of PR genes by the 2,4-D treatment

Transcription of *VvPR3*, *VvPR8* and of the three *VvPR10* genes was detected at the beginning of the experiment, suggesting a basal expression of these genes in embryos, although at a very low rate for *VvPR8*. However, *VvPR1* transcription was not detected at all. As a result of the 2,4-D treatment, *VvPR1*, *VvPR8*, *VvPR10.1* and *VvPR10.3* were strongly up-regulated, while *VvPR10.2* was only weakly stimulated and *VvPR3* not affected at all. The induction of some defence reactions is not surprising; 2,4-D is known to trigger an oxidative stress in treated tissues [60]. In a recent proteomic study, grapevine cultures strongly expressed varied defence related proteins after a treatment with 2,4-D [41]. Although not really understood, the relation between stress and SE is well documented, SE being also obtained, in some plant species, using non-hormonal inducers like wounding, high salt concentration, heavy metal ions or osmotic shock [16,53]. In fact, SE is thought to be an adaptative process to stress, resulting from the extensive cellular reorganization and the genetic reprogramming of treated tissues [16]. However, we found that *VvPR3*, encoding a class I chitinase, was not very responsive to the 2,4-D treatment, contrary to *VvPR8*, encoding a class III chitinase. Likewise, *VvPR10.1* and *VvPR10.3* were strongly up-regulated after the 2,4-D application, but not *VvPR10.2*. These results highlight a differential response to 2,4-D of *PR* genes encoding proteins with a similar enzymatic function. This further suggests that the two genes encoding chitinases (*VvPR3* and *VvPR8*) as well as the three *VvPR10* genes could be regulated in different ways and have separate roles in grapevine.

PR3 (a class I chitinase) is a basic enzyme located in the vacuole, whereas PR8 (a class III chitinase) is acidic and secreted to the apoplast. Plant chitinases can hydrolyse the β-1,4-glycoside bond present in biopolymers of N-acetylglucosamine, such as fungal chitin, as well as plant endogenous AGPs, releasing oligosaccharides that could play a role as signal molecules involved either in plant defence or in plant development [32]. PR3 and PR8 proteins have already been studied in different grapevine cultivars and their induction tested either upon pathogen attack or chemical treatment. According to these previous studies and despite complex patterns of response depending on the treatment and tissue type, general features may be drawn. PR8 is clearly related to grapevine SAR (Systemic Acquired Resistance), being induced by the chemical inducers SA (Salicylic Acid) and INA (2,6-dichloroIsoNicotinic Acid) [4], as well as during the incompatible interaction with *Pseudomonas syringae* [58]. On the contrary, PR3 seems to be constitutive, being expressed in untreated leaves and induced by wounding but not by SA or INA [6, 58]. This class I chitinase would not therefore be a marker of the SAR, although weakly induced under some pathogenic situations [6]. Consistent with these results, we found that *VvPR3* was continuously expressed during the whole culture, whereas *VvPR8* behaved as a typical defence gene, being induced by the 2,4-D stress.

A different expression profile was also found for *PR10* genes: *VvPR10.1* and *VvPR10.3* were strongly induced by the 2,4-D treatment but not *VvPR10.2*. PR10 is a unique group of intracytoplasmic small proteins often constitutively synthesized and thought to play a role in plant development and growth; they are also produced under pathogen attack and in response to abiotic stress [38]. In grapevine, *VvPR10.1* was shown to be up-regulated during an incompatible interaction of the cultivar 'Ugni blanc' with *P. syringae* [57], while several *PR10* genes were expressed in 'Cabernet-Sauvignon' following a fungal infection [19]. A PR10 protein closely related to *V. quinquangularis* PR10.3 was highly expressed in 'Riesling' leaves infected with *Plasmopara viticola* [52]. Moreover, PR10 proteins were produced at a very high level in grapevine plants submitted to a salt stress [31] or treated with the herbicide flumioxazin [5]. In our experiment, *VvPR10.2* was continuously expressed during the entire SE process, being only weakly up-regulated by the 2,4-D treatment. By contrast, *VvPR10.1* and *VvPR10.3* were strongly induced, as expected for defence genes.

Not detected in cotyledonary embryos at the beginning of the protocol, *VvPR1* expression was highly induced by the 2,4-D treatment. PR1 is a dominant group of PR proteins commonly used as markers of the SAR in many plants, despite a limited antifungal activity [69]. We previously showed that *VvPR1* is induced by SA but not under pathogen attack in whole grapevine plants, suggesting that it is not a good marker of grapevine SAR [6]. Other authors have reported a continuous expression of *VvPR1* in cell cultures and in in vitro plantlets but not in greenhouse plants, suggesting induction by a kind of 'in vitro stress' [71]. In the present study, *VvPR1* transcription was detected in calli after the 2,4-D treatment, but neither in embryos nor in calli at the last culture steps, showing that it was not a response to an 'in vitro stress'. Although both located on the chromosome 3, the *VvPR1* gene we monitored (AJ536326) and the one studied by Wielgoss and Kortekamp [71] (AJ003113) correspond to Genoscope annotations, respectively GSVIVT00038582001 and GSVIVT00038576001, bearing sufficient differences to be considered as separate genes. Three different PR1 proteins have been previously found to be induced by chemicals, wounding, as well as upon pathogen attack, therefore suggesting that several different *VvPR1* genes could be active in grapevine [56].

### 3.5. Putative role of PR proteins in SE

After the first culture step on a medium containing 2,4-D, all *PR* genes were expressed at a high rate, as a probable sign of oxidative stress. *VvPR1* and *VvPR8* were only transiently expressed after the

2,4-D treatment. Therefore, their role in the embryogenic process remains unclear. By contrast, *VvPR10.1* and *VvPR10.3* were expressed long after the 2,4-D removal, together with *VvPR3* and *VvPR10.2*. The class I chitinase encoded by *VvPR3* as well as the three PR10 proteins could therefore play a role during SE induction. In plant cell cultures, many different proteins are secreted; some of them are chitinase-like proteins, that have been shown to stimulate SE, for instance in grapevine, chicory, carrot, *Coffea arabica* and Norway spruce [13,27,59,68,72]. Plant chitinases may act by hydrolysing endogenous proteoglycans attached to the membranes or cell walls, such as AGPs, producing signal molecules that could have a function in SE [32]. A class IV chitinase, able to release oligosaccharides from cell AGPs, was shown to rescue the carrot *tsl1* mutant, impaired in embryo development [11,68]. Likewise, PR10 proteins would be able to condition cell cultures for SE, probably by interfering with the intracellular transport of hormones [17,33,38,40,45]. The overexpression of a *PR10* gene in a transgenic pea led to an elevated cytokinin content and a decreased abscisic acid level, suggesting that changes in the PR10 content could be relevant for phytohormone regulation [66]. In *M. truncatula*, some PR10 have been shown to be the most abundant proteins induced by a 2,4-D treatment leading to SE [12,30]. Moreover, a *PR10* gene was highly expressed during SE induction, starting from alfalfa protoplasts: a first pulse of transcription followed 2,4-D application and was interpreted as a stress response; the second pulse occurred later, at the time of embryo differentiation, suggesting an additional role of the encoded protein in morphogenesis [14]. However, PR10 expression was also recorded, during SE induction, in a highly (2HA) as well as in a poorly (Jemalong) embryogenic cell line of *M. truncatula*, suggesting that PR10 proteins could contribute to the conditioning of cultures but would not act alone for inducing the shift to embryogenesis [30]. In our SE model, all *PR* genes were expressed in the same way in embryogenic and non-embryogenic calluses, showing that both callus types had similarly been conditioned during the culture. A change was only observed in embryogenic clusters: *VvPR10.1* and *VvPR10.3* were down-regulated, while *VvSERK1*, *VvSERK2* and *VvL1L* were up-regulated, as the sign of the starting up of a new genetic program. In a proteomic analysis of SE, in the grapevine cultivar 'Thompson seedless', two forms of a PR10 protein were also down-regulated in young embryogenic clusters, whereas highly expressed in the underlying callus [41]. We found that *VvPR10.1* and *VvPR10.3* behaved differently from the other *PR* genes in our culture model. They were up-regulated by the 2,4-D treatment, being expressed for a long time in calli before the differentiation of secondary embryos. This suggests a possible specific role of the corresponding proteins in conditioning grapevine cultures for SE.

## 4. Materials and methods

### 4.1. Induction of secondary embryogenesis

Embryogenic cultures of *V. vinifera* cultivar 'Chardonnay' were previously obtained from nodal explants and maintained as described [39]. Culture media contained Murashige and Skoog [47] half strength major salts, micro-salts and vitamins, sucrose, and a mixture of growth regulators added before autoclaving. The pH level was adjusted to 5.8 by adding 1N NaOH. Media were solidified by adding 0.7% Bacto-Agar and sterilized by autoclaving at 115° C for 30 min. Culture incubation was performed at 25° C, in the dark. To induce secondary embryogenesis, intact and well-formed embryos were picked-up from embryogenic cultures. To assess secondary embryogenesis efficiency, four independant experiments were carried out, with embryos either at the torpedo-stage (experiments A and B) or at the cotyledonary-stage (experiments C and D). For molecular analyses, secondary embryogenesis was induced from cotyledonary embryos. Embryos were separately plated onto the medium E96 containing 60 g $L^{-1}$ sucrose, 9 μM BAP (6-BenzylAminoPurine) and 4.5 μM 2,4-D. A set of 14 embryos was placed in each Petri dish (60 mm of diameter). Cultures were incubated for 3 weeks to produce calli. Subculture was performed by transferring the calli onto the medium A containing 60 g $L^{-1}$ sucrose, 2.5 g $L^{-1}$ activated charcoal, 20 μM IAA (Indole-3-Acetic Acid), 10 μM NOA (2-NaphtOxyacetic Acid) and 1 μM BAP. Transfer onto a fresh medium occurred every 4 weeks until clusters of secondary embryos appeared on calli. For germination of secondary embryos, embryos at the cotyledonary-stage were transferred, 3 per Petri dish, onto a medium containing 25 g $L^{-1}$ sucrose and 0.2 μM BAP, and incubated for 4–8 days.

### 4.2. Collecting of tissues for molecular analysis

Tissues were collected at different steps of the secondary embryogenesis process: cotyledonary embryos at the beginning of the experiment (E); calli developed from embryos on the medium E96 (C1); calli after a one week subculture on the medium A (C2); non-embryogenic calli (NEC) and embryogenic calli cleared of embryogenic clusters (EC) after 3 subcultures on the medium A; and clusters of secondary embryos (CSE). Germinated embryos (GE) bearing a visible apical meristem, two developed cotyledons and a differentiated root were also collected. Samples were composed of pools of embryos or calli, carefully harvested and freezed in liquid $N_2$ for preserving at −80° C.

### 4.3. RNA extraction

Total RNA was extracted using the 'Nucleospin RNA plant kit' (Macherey-Nagel, Germany) following the manufacturer's instructions with slight modifications: samples corresponding to around 100 mg fresh tissue were ground in liquid nitrogen and transferred into 500 μL buffer RA1 after the addition of 2.5% PVP 40, 1% β-mercaptoethanol and 2% sarkosyl (chemicals purchased from Sigma–Aldrich, Germany). Incubation was performed at 70° C for 10 min. Total RNA was quantified by Optical Density measurement. The absence of contaminating genomic DNA was checked by a PCR test with the total RNA extract as a template and *VvActin* primers.

### 4.4. Semi-quantitative RT-PCR analysis

RT-PCR amplifications were performed using the 'Superscript II First-Strand Synthesis system' (Invitrogen, USA), starting from 1 μg total RNA. cDNA synthesis was performed at 50° C for 60 min and then 70° C for 15 min. Amplifications were carried out according to the following program: 30 s at 95° C (denaturation), 30 s at 55° C (annealing) and 1 min at 72° C (extension), with a final elongation step at 72° C for 7 min. Prior to expression analysis, *VvActin* was used as a standard to determine the exponential zone of amplification in a PCR test of 28 cycles with 5 serial half-dilutions of each cDNA sample. The appropriate dilutions were then used in PCR amplifications of 32 cycles. Three independent PCR reactions were performed for each gene, giving rise to very reproducible results. We used primer sequences corresponding to the different genes and deduced from the nucleotide sequences available in the banks of the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov) and the Genoscope (http://www.genoscope.cns.fr): forward 5′-TGC TAT CCT TCG TCT TGA CCT TG-3′ and reverse 5′-GGA CTT CTG GAC AAC GGA ATC TC-3′ for *VvActin* (AF369525), forward 5′-GCC TAC GCC CAG AAC TAT GCT AAC-3′ and reverse 5′-CGA ACC ACC ACC CAT TGT TG-3′ for *VvPR1* (AJ536326),

forward 5′-TGT AGC AAC TAA GAC GTT TAT TCA-3′ and reverse 5′-GCC AGC TTA TTA CCA GGT CCA T-3′ for *VvPR3* (AJ291505), forward 5′-ATC ATC GTC TCG GCC ATT AG-3′ and reverse 5′-AGA GCA GTG CCC ATG AAC TT-3′ for *VvPR8* (AJ291507), forward 5′-CAA CCA CAG TGT AGC TGA ATG TGA AG-3′ and reverse 5′-CGA GAG TGA GGT CAC TTC CTC G-3′ for *VvPR10.1* (AJ291705), forward 5′-CGA TCA CAG TGT AGC GGA ATG AGA AT-3′ and reverse 5′-AAG CTA TCA AGT GCG TGG AAG TCA TT-3′ for *VvPR10.2* (AJ291704), forward 5′-TGT TTA AGA GAA CGC CC-3′ and reverse 5′-GAG TTG GAG TGA GGA GCG-3′ for *VvSERK1* (GSVIVT00001777001), forward 5′-GCC TAA GAG AAC GTC CAC CAT-3′ and reverse 5′-ACC TCC TGA CGG ACG ACC-3′ for *VvSERK2* (GSVIVT00019412001), forward 5′-GTT TGA GAG AGC GAG CTG ATG-3′ and reverse 5′-GGT GGG GGA TAT GGT TGT A-3′ for *VvSERK3* (GSVIVT00009544001), forward 5′-CAG CCA TGG AAG ACA CTG AAT GCA-3′ and reverse 5′-CCT TCA AGC TCG CGG TAG TGG TGG-3′ for *VvL1L* (GSVIVT00010958001). The resulting amplification products were analyzed by electrophoresis on a 1.5 % agarose gel and stained with ethidium bromide. Sequencing of the PCR products was performed in order to determine the amplified sequences (Genoscreen, France). Nucleic acid homology search was performed through the databases of the NCBI, the Genoscope, the Istituto Agrario di San Michele all'Adige (IASMA) (http://genomics.research.iasma.it) and the Dana-Farber Harvard Cancer Institute (DFCI) (http://compbio.dfci.harvard.edu). Sequence alignments were performed using the program ClustalW of the European Bioinformatics Institute (EBI) (http://ebi.ac.uk).

### 4.5. Rapid amplification of cDNA ends (RACE)

Further determination of a cDNA sequence amplified with the *VvPR10.1* primers was performed using a RACE-PCR protocol with oligonucleotides allowing to extend it in both directions (toward the 5′ and the 3′ regions), and leading to slightly overlapping sequences. RACE samples were prepared from the poly(A)$^+$ RNA using the "5′/3′ RACE Kit" (Roche, France), following the manufacturer's instructions. 5′RACE required three antisens specific primers: 5′-GGG ACC TCC CTG TCC TTG TAG-3′ (SP1); 5′-CTT GAG GCC TTA TCT TGG G-3′ (SP2) ; 5′-CGA GGA AGT GAC CTC ACT CTC G-3′ (SP3), whereas 3′RACE required only one specific forward primer: 5′-CTA CAA GGA CAG GGA GGT CCC-3′ (SP5).

## References


[1] L. Alemanno, M. Devic, N. Niemenak, C. Sanier, J. Guilleminot, M. Rio, J.-L. Verdeil, P. Montoro, Characterization of *leafy cotyledon1-like* during embryogenesis in *Theobroma cacao* L, Planta 227 (2008) 853–866.

[2] L. Beuning, J.H. Bowen, H.A. Persson, D. Barraclough, S. Bulley, E.A. MacRae, Characterisation of Mal d 1-related genes in *Malus*, Plant Mol. Biol. 55 (2004) 369–388.

[3] H. Breiteneder, K. Pettenburger, A. Bito, R. Valenta, D. Kraft, H. Rumpold, O. Scheiner, M. Breitenbach, The gene coding for the major birch pollen allergen, Bet v. 1, is highly homologous to a pea disease resistance response gene, EMBO J. 8 (1989) 1935–1938.

[4] G. Busam, H.H. Kassemeyer, U. Matern, Differential expression of chitinases in *Vitis vinifera* L. responding to systemic acquired resistance activators or fungal challenge, Plant Physiol. 115 (1997) 1029–1038.

[5] A.J. Castro, C. Carapito, N. Zorn, C. Magné, E. Leize, A. Van Dorsselaer, C. Clément, Proteomic analysis of grapevine (*Vitis vinifera* L.) tissues subjected to herbicide stress, J. Exp. Bot. 56 (2005) 2783–2795.

[6] J. Chong, G. Le Henanff, C. Bertsch, B. Walter, Identification, expression analysis and characterization of defense signaling genes in *Vitis vinifera*, Plant Physiol. Biochem. 46 (2008) 469–481.

[7] A. Chugh, P. Khurana, Gene expression during somatic embryogenesis-recent advances, Curr. Sci. 83 (2002) 715–730.

[8] J. Colcombet, A. Boisson-Dernier, R. Ros-Palau, C.E. Vera, J.I. Schroeder, *Arabidopsis* SOMATIC EMBRYOGENESIS RECEPTOR KINASES1 and 2 are essential for tapetum development and microscope maturation, Plant Cell 17 (2005) 3350–3361.

[9] P. Croce, C. Vannini, M. Bracale, An easy and convenient method for maintenance of embryogenic cultures of *Vitis vinifera*, Vitis 44 (2005) 197–198.

[10] D.K. Das, M.K. Reddy, K.C. Upadhyaya, S.K. Sopory, An efficient leaf-disc culture method for the regeneration via somatic embryogenesis and transformation of grape (*Vitis vinifera* L.), Plant Cell Rep. 20 (2002) 999–1005.

[11] A.J. De Jong, J. Cordewener, F. Lo Schiavo, M. Terzi, J. Vandekerckhove, A. Van Kammen, S.C. De Vries, A carrot somatic embryo mutant is rescued by chitinase, Plant Cell 4 (1992) 425–433.

[12] F. De Jong, U. Mathesius, N. Imin, B.G. Rolfe, A proteome study of the proliferation of cultured *Medicago truncatula* protoplasts, Proteomics 7 (2007) 722–736.

[13] A. Deloire, E. Kraeva, M.C. Mauro, E. Bonnet, J. Bierne, Immunodetection of chitinase-like and β-1,3-glucanase-like proteins secreted in vitro by embryogenic and non-embryogenic cells of grapevines, Vitis 1 (1997) 51–52.

[14] M. Domoki, J. Györgyey, J. Biro, T.P. Pasternak, A. Zvara, S. Bottka, L.G. Puskas, D. Dudits, A. Fehér, Identification and characterization of genes associated with the induction of embryogenic competence in leaf-protoplast-derived alafalfa cells, Biochim. Biophys. Acta 1759 (2006) 543–551.

[15] M. Fambrini, C. Durante, G. Cionini, C. Geri, L. Giorgetti, V. Michelotti, M. Salvini, C. Pugliesi, Characterization of *LEAFY COTYLEDON1-LIKE* gene in *Helianthus annuus* and its relationship with zygotic and somatic embryogenesis, Dev. Genes Evol. 216 (2006) 253–264.

[16] A. Fehér, T.P. Pasternak, D. Dudits, Transition of somatic plant cells to an embryogenic state, Plant Cell Tissue Organ Cult. 74 (2003) 201–228.

[17] H. Fernandes, O. Pasternak, G. Bujacz, A. Bujacz, M. Sikorski, M. Jaskolski, *Lupinus luteus* Pathogenesis-related protein as a reservoir for cytokinin, J. Mol. Biol. 378 (2008) 1040–1051.

[18] C. Finkler, C. Giacomet, V.C. Muschner, F.M. Salzano, L.B. Freitas, Molecular investigations of pathogenesis-related Betv1 homologues in *Passiflora* (Passifloraceae), Genetica 124 (2005) 117–125.

[19] R.W.M. Fung, M. Gonzalo, C. Fekete, L.G. Kovacs, Y. He, E. Marsh, L.M. McIntyre, D.P. Schachtman, W. Qiu, Powdery mildew induces defense-oriented reprogramming of the transcriptome in a susceptible but not in a resistant grapevine, Plant Physiol. 146 (2008) 236–249.

[20] M.D. Gaj, Direct somatic embryogenesis as a rapid and efficient system for *in vitro* regeneration of *Arabidopsis thaliana* (L.) Heynh, Plant Cell Tissue Organ Cult. 64 (2001) 39–46.

[21] M.D. Gaj, Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh, Plant Growth Regul. 43 (2004) 27–47.

[22] M.D. Gaj, S. Zhang, J.J. Harada, P.G. Lemaux, Leafy cotyledon genes are essential for induction of somatic embryogenesis of *Arabidopsis*, Planta 222 (2005) 977–988.

[23] G. Gambino, P. Ruffa, R. Vallania, I. Gribaudo, Somatic embryogenesis from whole flowers, anthers and ovaries of grapevine (*Vitis* spp.), Plant Cell Tissue Organ Cult. 90 (2007) 79–83.

[24] V. Hecht, J.P. Vielle-Calzada, M.V. Hartog, E.D.L. Schmidt, K. Boutilier, U. Grossniklaus, S.C. de Vries, The Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR KINASE1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture, Plant Physiol. 127 (2001) 803–816.

[25] A. Heese, D.R. Hann, S. Gimenez-Ibanez, A.M.E. Jones, K. He, J. Li, J.I. Schroeder, S.C. Peck, J.P. Rathjen, The receptor-like kinase *SERK3*/*BAK1* is a central regulator of innate immunity in plants, Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 12217–12222.

[26] S. Helleboid, G. Bauw, L. Belingheri, J. Vasseur, J.L. Hilbert, Extracellular β-1,3-glucanases are induced during early somatic embryogenesis in *Cichorium*, Planta 205 (1998) 56–63.

[27] S. Helleboid, T. Hendricks, G. Bauw, D. Inzé, J. Vasseur, J.L. Hilbert, Three major somatic embryogenesis related proteins in *Cichorium* identified as PR proteins, J. Exp. Bot. 51 (2000) 1189–1200.

[28] K. Hoffmann-Sommergruber, M. Vanek-Krebitz, C. Radauer, J. Wen, F. Ferreira, O. Scheiner, H. Breiteneder, Genomic characterization of members of the Bet v 1 family: genes coding for allergens and pathogenesis-related proteins share introns positions, Gene 197 (1997) 91–100.

[29] H. Hu, L. Xiong, Y. Yang, Rice *SERK1* gene positively regulates somatic embryogenesis of cultured cells and host defense response against fungal infection, Planta 222 (2005) 107–117.

[30] N. Imin, M. Nizamidin, D. Daniher, K.E. Nolan, R.J. Rose, B.G. Rolfe, Proteomic analysis of somatic embryogenesis in *Medicago truncatula*. Explant cultures grown under 6-benzylaminopurine and 1-naphtaleneacetic acid treatments, Plant Physiol. 137 (2005) 1250–1260.

[31] N. Jellouli, H. Ben Jouira, H. Skouri, A. Ghorbel, A. Gourgouri, A. Mliki, Proteomic analysis of tunisian grapevine cultivar Razegui under salt stress, J. Plant Physiol. 165 (2008) 471–481.

[32] A. Kasprzewska, Plant chitinases–regulation and function, Cell. Mol. Biol. Lett. 8 (2003) 809–824.

[33] K.M. Koistinen, P. Soininen, T.A. Venäläinen, J. Häyrinen, R. Laatikainen, M. Peräkylä, A.I. Tervahauta, S.O. Kärenlampi, Birch PR-10c interacts with several biologically important ligands, Phytochemistry 66 (2005) 2524–2533.

[34] M.A.C.J. Kwaaitaal, S.C. de Vries, The *SERK1* gene is expressed in procambium and in immature vascular cells, J. Exp. Bot. 58 (2007) 2887–2896.

[35] R.W. Kwong, A.Q. Bui, H. Lee, L.W. Kwong, R.L. Fischer, R.B. Goldberg, J.J. Harada, LEAFY COTYLEDON1-LIKE defines a class of regulators essential for embryo development, Plant Cell 15 (2003) 5–18.

[36] S. Legrand, T. Hendricks, J.L. Hilbert, M.C. Quillet, Characterization of expressed sequence tags obtained by SSH during somatic embryogenesis in *Cichorium intybus* L, BMC Plant Biol. 7 (2007) 27.

[37] J. Li, J. Wen, K.A. Lease, J.T. Doke, F.E. Tax, J.C. Walker, BAK1, an *Arabidopsis* LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling, Cell 110 (2002) 213–222.
[38] J.J. Liu, A.K.M. Ekramoddoullah, The family 10 of plant pathogenesis-related proteins: their structure, regulation, and function in response to biotic and abiotic stresses, Physiol. Mol. Plant Pathol. 68 (2006) 3–13.
[39] P. Maillot, F. Kieffer, B. Walter, Somatic embryogenesis from stem nodal sections of grapevine, Vitis 45 (4) (2006) 185–189.
[40] Z. Markovic-Housley, M. Degano, D. Lamba, E. von Roepenack-Lahaye, S. Clemens, M. Susani, F. Ferreira, O. Scheiner, H. Breiteneder, Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant sterol carrier, J. Mol. Biol. 325 (2003) 123–133.
[41] M. Marsoni, M. Bracale, L. Espen, B. Prinsi, A.S. Negri, C. Vannini, Proteomic analysis of somatic embryogenesis in *Vitis vinifera*, Plant Cell Rep. 27 (2008) 347–356.
[42] L. Martinelli, E. Candioli, D. Costa, V. Poletti, N. Rascio, Morphogenic competence of *Vitis rupestris* S. secondary somatic embryos with a long culture history, Plant Cell Rep. 20 (2001) 279–284.
[43] L. Martinelli, I. Gribaudo, D. Bertoldi, E. Candioli, V. Poletti, High efficency somatic embryogenesis and plant germination in grapevine cultivars Chardonnay and Brachetto a grappolo lungo, Vitis 40 (2001) 111–115.
[44] L. Martinelli, G. Mandolino, Transgenic grapes (*Vitis* species, in: Y.P.S. Bajaj (Ed.), Biotechnology in Agriculture and Forestry, Springer-Verlag, Berlin, 2001, pp. 325–338.
[45] J.E. Mogensen, R. Wimmer, J.N. Larsen, M.D. Spangfort, D.E. Otzen, The major birch allergen, Bet v 1, shows afinity for a broad spectrum of physiological ligands, J. Biol. Chem. 277 (2002) 23684–23692.
[46] C. Morgana, R. Di Lorenzo, F. Carimi, Somatic embryogenesis of *Vitis vinifera* L. (cv. Sugraone) from stigma and style culture, Vitis 43 (2004) 169–173.
[47] T. Murashige, F. Skoog, A revised medium for the rapid growth and bioassays with tobacco tissue culture, Physiol. Plant. 15 (1962) 473–497.
[48] R.R. Nair, S. Dutta Gupta, High-frequency plant regeneration through cyclic secondary somatic embryogenesis in black pepper (*Piper nigrum*) L, Plant Cell Rep. 24 (2006) 699–707.
[49] K.H. Nam, J. Li, BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling, Cell 110 (2002) 203–212.
[50] K.E. Nolan, R.R. Irwanto, R.J. Rose, Auxin up-regulates *MtSERK1* expression in both *Medicago truncatula* root-forming and embryogenic cultures, Plant Physiol. 133 (2003) 218–230.
[51] M. Perrin, C. Gertz, J.E. Masson, High efficiency initiation of regenerable embryogenic callus from anther filaments of 19-grapevine genotypes grown worldwide, Plant Sci. 167 (2004) 1343–1349.
[52] M. Polesani, F. Desario, A. Ferrarini, A. Zamboni, M. Pezzotti, A. Kortekamp, A. Polverari, cDNA-AFLP analysis of plant and pathogen genes expressed in grapevine infected with *Plasmopara viticola*, BMC Genomics 9 (2008) 142.
[53] F.R. Quiroz-Figueroa, R. Rojas-Herrera, R.M. Galaz-Avalos, V.M. Loyola-Vargas, Embryo production through somatic embryogenesis can be used to study cell differentiation in plants, Plant Cell Tissue Organ Cult. 86 (2006) 285–301.
[54] C.J.J.M. Raemakers, E. Jacobsen, R.G.F. Visser, Secondary somatic embryogenesis and applications in plant breeding, Euphytica 81 (1995) 93–107.
[55] V. Raghavan, Role of 2,4-Dichlorophenoxyacetic acid (2,4-D) in somatic embryogenesis on cultured zygotic embryos of *Arabidopsis*: cell expansion, cell cycling, and morphogenesis during continuous exposure of embryos to 2,4-D, Am. J. Bot. 91 (2004) 1743–1756.
[56] V. Repka, J. Kubikova, I. Fischerova, Immunodetection of PR1-like proteins in grapevine leaves infected with *Oidium tuckeri* and in elicited suspension cultures, Vitis 39 (2000) 123–127.
[57] N. Robert, J. Ferran, C. Breda, P. Coutos-Thévenot, M. Boulay, D. Buffard, R. Esnault, Molecular characterization of the incompatible interaction of *Vitis vinifera* leaves with *Pseudomonas syringae* pv. *pisi*: Expression of genes coding for stilbene synthase and class 10 PR protein, Eur. J. Plant Pathol. 107 (2001) 249–261.
[58] N. Robert, K. Roche, Y. Lebeau, C. Breda, M. Boulay, R. Esnault, D. Buffard, Expression of grapevine chitinase genes in berries and leaves infected by fungal or bacterial pathogens, Plant Sci. 162 (2002) 389–400.
[59] R. Rojas-Herrera, V.M. Loyola-Vargas, Induction of a class III chitinase in foliar explants of *Coffea arabica* L. during somatic embryogenesis and wounding, Plant Sci. 163 (2002) 705–711.
[60] M.C. Romero-Puertas, I. McCarthy, M. Gomez, L.M. Sandalio, F.J. Corpas, L.A. Del Rio, J.M. Palma, Reactive oxygen species-mediated enzymatic systems involved in the oxidative action of 2,4-dichlorophenoxyacetic acid, Plant Cell Environ. 27 (2004) 1135–1148.
[61] M.O. Santos, E. Romano, K.S.C. Yotoko, M.L.P. Tinoco, B.B.A. Dias, F.J.L. Aragao, Characterisation of the cacao *somatic embryogenesis receptor-like kinase* (*SERK*) gene expressed during somatic embryogenesis, Plant Sci. 168 (2005) 723–729.
[62] P. Schellenbaum, A. Jacques, P. Maillot, C. Bertsch, F. Mazet, S. Farine, B. Walter, Characterization of *VvSERK1, VvSERK2, VvSERK3* and *VvL1L* genes and their expression during somatic embryogenesis of grapevine (*Vitis vinifera* L.), Plant Cell Rep. 27 (2008) 1799–1809.
[63] E.D.L. Schmidt, F. Guzzo, M.A.J. Toonen, S.C. de Vries, A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos, Development 124 (1997) 2049–2062.
[64] B. Singla, A.K. Tyagi, J.P. Khurana, P. Khurana, Analysis of expression profile of selected genes expressed during auxin-induced somatic embryogenesis in leaf base system of wheat (*Triticum aestivum*) and their possible interactions, Plant Mol. Biol. 65 (2007) 677–692.
[65] M.N. Somleva, E.D.L. Schmidt, S.C. de Vries, Embryogenic cells in *Dactylis glomerata* L. (Poaceae) explants identified by cell tracking and by SERK expression, Plant Cell Rep. 19 (2000) 718–726.
[66] S. Srivastava, R.J. Neil Emery, L.V. Kurepin, D.M. Reid, B. Fristensky, N.N.V. Kav, Pea PR10.1 is a ribonuclease and its transgenic expression elevates cytokinin levels, Plant Growth Regul. 49 (2006) 17–25.
[67] C. Thomas, D. Meyer, C. Himber, A. Steinmetz, Spatial expression of a sunflower *SERK* gene during induction of somatic embryogenesis and shoot organogenesis, Plant Physiol. Biochem. 42 (2004) 35–42.
[68] A.J. Van Hengel, Z. Tadesse, P. Immerzeel, H. Schols, A. van Kammen, S.C. de Vries, N-Acetylglucosamine and glucosamine-containing arabinogalactan proteins control somatic embryogenesis, Plant Physiol. 125 (2001) 1880–1890.
[69] L.C. Van Loon, E.A. Van Strien, The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins, Physiol. Mol. Plant Pathol. 55 (1999) 85–97.
[70] J. Wen, M. Vanek-Krebitz, K. Hoffmann-Sommergruber, O. Scheiner, H. Breiteneder, The potential of *Betv1* homologues, a nuclear multigene family, as phylogenetic markers in flowering plants, Mol. Phylogenet. Evol. 3 (1997) 317–333.
[71] A. Wielgoss, A. Kortekamp, Comparison of *PR1* expression in grapevine cultures after inoculation with a host- and a non-host pathogen, Vitis 45 (2006) 9–13.
[72] M. Wiweger, I. Farbos, M. Ingouff, U. Lagercrantz, S. von Arnold, Expression of Chia4-Pa chitinase genes during somatic and zygotic embryo development in Norway spruce (*Picea abies*): similarities and differencies between gymnosperm and angiosperm class IV chitinases, J. Exp. Bot. 54 (2003) 2691–2699.